APPENDIX 5

ACCELERATED COMMUNICATION

Loss of Nucleotide Regulation of Epithelial Chloride Transport in the Jejunum of P2Y$_4$-Null Mice

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ABSTRACT

The P2Y$_4$ receptor is responsive to UTP in human and to ATP and UTP in rodents. With the aim of identifying its pharmaco-therapeutic interest, we generated P2Y$_4$-null mice by a classic gene targeting method. The proportion of genotypes was consistent with X-linked Mendelian transmission. Gene inactivation was checked by the complete disappearance of P2Y$_4$ receptor mRNA from liver, stomach, and intestine. The P2Y$_4$-null mice had a grossly normal behavior, growth, and reproduction. Chloride secretion by the jejunal epithelium was assessed in Ussing chambers by the measurement of the short circuit current in the presence of phlorizin. We show here that the UTP- and ATP-induced chloride secretory responses observed in wild-type mice are abolished in P2Y$_4$-null mice. This is the first clearcut demonstration of a biological role of the P2Y$_4$ receptor.

The P2Y$_4$ receptor is a member of the P2Y family of G-protein-coupled receptors for extracellular nucleotides. Cloning of the human P2Y$_4$ receptor was reported in 1995 (Communi et al., 1995; Nguyen et al., 1995; Stam et al., 1996) and was followed by the cloning of the rat (Bogdanov et al., 1998; Webb et al., 1998) and mouse (Lazarowski et al., 2001; Suarez-Huerta et al., 2001) orthologs. The human P2Y$_4$ receptor is a selective UTP receptor, whereas the rodent ones are activated equipotently by UTP and ATP. No physiological role of the P2Y$_4$ receptor has yet been established, and its pharmacotherapeutic potential thus remains uncertain. P2Y$_4$ mRNA has been detected in human placenta (Communi et al., 1995) and in murine stomach, intestine, and liver (Suarez-Huerta et al., 2001). In the adult rat, they were detected in multiple organs but at a low level, whereas the expression was higher in neo-natal animals (Webb et al., 1998). The presence of P2Y$_4$ mRNA and/or protein, combined with functional responses to UTP, supports the expression of the P2Y$_4$ receptor in 6CFSMEo- submucosal cells derived from human lung (Communi et al., 1999), in the vestibular dark cell epithelium of gerbil inner ear (Marcus and Scofield, 2001; Sage and Marcus, 2002), and in rat aortic smooth muscle cells (Harper et al., 1998). Expression in endothelial cells (Jin et al., 1998) has also been reported and might be related to the chemotactic and mitogenic actions of UTP on guinea pig coronary endothelial cells (Satterwhite et al., 1999). In addition, the persistence of a Cl$^-$ secretory response to UTP and ATP in P2Y$_2$$^{-/-}$ mice led to the suggestion that this response could be mediated by the P2Y$_4$ receptor (Cressman et al., 1999). To evaluate the physiological role and pharmacotherapeutic potential of the P2Y$_4$ receptor we have generated P2Y$_4$-deficient mice. In the work reported here, we focused our attention on the regulation by ATP and UTP of the Cl$^-$ secretion in the jejunal epithelium with the aim of identifying the P2Y receptor mediating this control.

ABBREVIATIONS: bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcription; HPRT, hypoxanthine phosphoribosyltransferase; SSC, short-circuit current; 8-p-SPT, 8-(para-sulfophenyl)theophylline; ES, embryonic stem; CFTR, cystic fibrosis transmembrane conductance regulator.

Materials and Methods

Generation of P2Y$_4$-Deficient Mice. To generate the targeting vector, a 5'-SalI-XbaI-3' 4900-bp fragment, with the XbaI site located 700 bp upstream from the initiation codon of the P2Y$_4$ gene, was inserted in the pFlox vector upstream from the neomycin gene (Fig. 1A). A 2300-bp fragment, beginning at base 399 of the coding sequence of the gene and obtained by PCR, was inserted in the BamHI and XhoI sites of the targeting plasmid (i.e., downstream from the neomycin resistance cassette). Upon integration of the targeting vector, 1100 bp of the recombinant locus containing the first 398 bp of the published P2Y$_4$ coding sequence are deleted.

After electroporation of this targeting vector in R1 ES cells, G-418–resistant colonies were isolated. Classically extracted genomic DNA was analyzed by EcoRI digestion followed by Southern blotting using a DNA probe located outside the targeting vector, downstream from the P2Y$_4$ gene. This probe detected a 7-kilobase pair fragment of the wild-type locus and a 8.6-kilobase pair fragment of the mutated locus.

R1 P2Y$_4$$^{+/-}$ ES cells were cocultured overnight with CD1 morulae. Blastulae were then transferred into CD1 pseudopregnant female mice to generate chimeric mice. Offspring genotype was analyzed by Southern blotting as described above or by PCR. The actual repartition of genotypes was compared with the theoretical repartition expected from a binomial distribution with equal frequency of each allele.

Total RNA Isolation, Reverse Transcription, and PCR Procedure. The expression of the P2Y$_4$ receptor mRNA was studied on tissues extracted from wild-type and P2Y$_4$-null male mice. Total mouse RNA was isolated from tested organs using TriPure reagent following the manufacturer's instructions. One microgram of total RNA was submitted to OneStep reverse transcription PCR according to the manufacturer's recommendations. Oligonucleotide amplification primers were designed according to the mouse P2Y$_4$ sequence: sense primer, 5'-gactccttgctattcacat-3'; antisense primer, 5'-agtagaggttccagtagaaa-3'. OneStep RT-PCR was performed with 0.6 μM concentrations of each primer, with Q solution, and with 0.6 units of RNase inhibitor under the following conditions: 30 min at 50°C for the reverse transcription, 15 min at 95°C for the inactivation of the reverse transcriptase and the initial PCR activation step, followed by 3-step cycles (30 or 40 times) of 60 s at 94°C, 60 s at 52°C, 60 s at 72°C, and a final extension of 10 min at 72°C. Genomic DNA contamination was checked by omitting reverse transcription.

The expression of HPRT mRNA was used for internal control of expression. Oligonucleotide amplification primers were designed according to the mouse HPRT sequence: sense primer, 5'-gctggtgaaaaggacctct-3'; antisense primer, 5'-cacaggactagaacacctgc-3'. The protocol was the same as reported above, except that the PCR conditions were: (30 s at 94°C, 45 s at 57°C, 60 s at 72°C) × 30 cycles, and a final extension of 10 min at 72°C.

Amplification products were resolved on 1.5% (w/v) agarose gel by electrophoresis and visualized under UV light after ethidium bromide coloration.

Bioelectric Measurements in Isolated Mouse Jejunum. The technique will be fully described in another article that will cover the theoretical aspect of the impedance analysis performed. Briefly, mice aged 2 to 5 months were sacrificed by intraperitoneal pentobarbital (10 mg/kg). The midportion of the jejunum, extending 10 cm after the ligament of Treitz, was dissected, opened, and washed with Krebs bicarbonate solution. The jejunal mucosa was stripped from the adjacent muscularis layer and sealed on the basolateral side to a fixation ring with an opening diameter of 3 mm. This ring was placed between the halves of an Ussing chamber. KCl electrodes connected to the solution via a short agar bridge were used for measuring potential difference and passing current. Impedance analysis was used to determine the resistance of the epithelium and bathing solution between the voltage electrodes. The impedance spectrum represented in a Nyquist diagram consisted of a single semicircle. This behavior is caused by the paracellular conductance related to the structure of the epithelium and/or possible damage of the epithelium caused by stripping the mucosa. Given these parameters, the epithelium can be represented by a lumped model consisting of a parallel circuit of a capacitance and resistance in series with the solution resistance between the voltage electrodes (R_{sol}). The resistance shunting the capacitance represents the transepithelial resistance (R_{epi}). In the Nyquist diagram, the intercepts of the semicircle at high and low frequencies correspond to R_{sol} and $R_{sol} + R_{epi}$, respectively. In this series of experiments, the mean values were: $R_{sol} = 25 \pm 3$ Ω/cm^2 and $R_{epi} = 14 \pm 2$ Ω/cm^2. R_{sol} attenuates the current recorded by the voltage clamp (SCC_{rec}), and the short circuit current (SCC) expected for an ideal voltage clamp across the epithelium can be calculated as: $SCC = SCC_{rec} (R_{epi} + R_{sol})/R_{epi}$. The volume of each compartment bathing the jejunal mucosa was 2 ml, and Krebs bicarbonate solution, pre-equilibrated with a gas mixture of 5% CO_2/95% O_2 at 37°C, was flowing in each compartment at a rate of 20 ml/min. The composition of the Krebs bicarbonate solution was 140 mM Na, 5.2 mM K, 1.2 mM Mg, 1.2 mM Ca, 120 mM Cl, 2.8 mM PO$_4$, 25 mM HCO$_3$, and 11.5 mM glucose, pH 7.4.

Study of Cl$^-$ Secretory Response in Jejunum. Preliminary experiments in jejuna from control mice indicated that the short-circuit current can be divided into two components: 1) a sodium absorptive component caused by the operation of the sodium-glucose cotransporter at the apical border of villus enterocytes and 2) a chloride secretory component linked to the existence of apical chloride channels in crypt cells. The first component could be eliminated by addition of 1 mM phlorizin to the apical bath, whereas the latter component was quantitatively accounted for by chloride secretion, because it was abolished in chloride-free solutions. Furthermore, the maneuvers of stripping the mucosa from its adjacent muscularis and mounting it in a small Ussing chamber induce the release of prostaglandins, which constitute a stimulus to chloride secretion, as first demonstrated by Pierce et al. (1971), and could therefore obscure other stimuli. Therefore, jejunal chloride secretion was assessed as the SSC after addition of phlorizin (1 mM) to the mucosal side as well as of indomethacin to both bathing media (100 μM). UTP or ATP were added at 100 μM to the apical solution and in some experiments

A

B

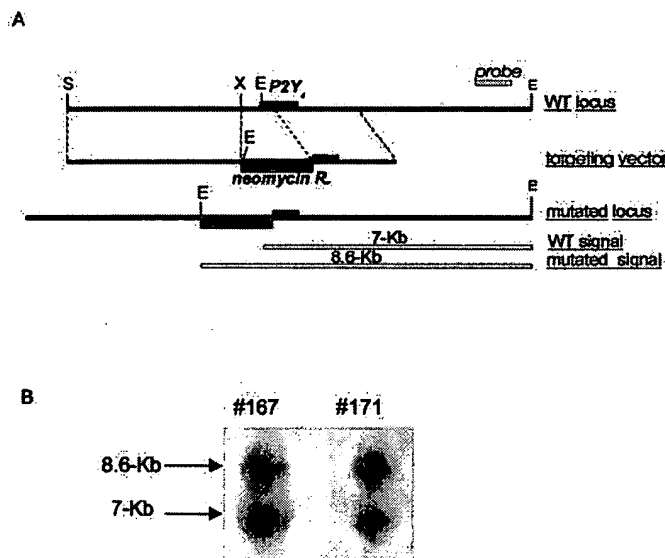Fig. 1. A, schematic representation of the targeting vector, endogenous allele and mutated allele after homologous integration. X, XbaI; E, EcoRI; S, SalI. B, Southern blot analysis of genomic DNA extracted from two recombinant clones of ES cells. After extraction, genomic DNA of ES clones was analyzed by EcoRI digestion and Southern blotting as described under Materials and Methods. The 7-kilobase pair signal corresponds to the wild-type allele and the 8.6-kilobase pair signal to the targeted allele.

after preincubation in the presence of 8-(para-sulfophenyl)theophylline (8-p-SPT). Forskolin was added to the basolateral solution at the concentration of 10 μM in ethanol 0.1%; this concentration of ethanol does not affect SCC (data not shown). The SCC was expressed in microamperes per square centimeter. Data are expressed as mean ± S.E.M. Statistical analysis was performed using unpaired t test.

Materials. OneStep reverse transcription PCR was from QIAGEN (Westburg, Leusden, The Netherlands). RNase inhibitor was from Invitrogen (Merelbeke, Belgium). TriPure kit was from Roche Diagnostics (Basel, Switzerland). UTP, ATP, adenosine, indomethacin, forskolin, phlorizin, and 8-p-SPT were purchased from Sigma (Merelbeke, Belgium).

R1 ES cells were a generous gift of Dr. A. Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). CD1 mice were purchased from Iffa Credo Belgium s.a. and Harlan France.

Results

Generation of P2Y₄-Null Mice. We generated a targeting vector to inactivate the $P2Y_4$ gene. Insertion of the neomycin resistance cassette upstream from the gene was associated with the deletion of 700 bp of the promoter region and of the first 399 nucleotides of the coding sequence (Fig. 1A; see *Materials and Methods* for details). Clones of R1 ES cells electroporated with the targeting vector were analyzed for homologous recombination by enzyme restriction and Southern blotting (Fig. 1B). P2Y₄$^{+/-}$ ES cells were then aggregated with morulae and chimeric male mice were crossed with wild-type CD1 female mice. Eighteen F1 newborn mice were obtained from two independent crosses: all female mice were P2Y₄$^{+/-}$ and all male mice were P2Y₄$^{+/+}$ (Fig. 2A). This observation was compatible with a location of the $P2Y_4$ gene on the X chromosome, as is the case for the human ortholog (Nguyen et al., 1995). Throughout this article, wild-type male mice will be referred to as P2Y₄$^{0/+}$ and mutated male mice as P2Y₄$^{0/-}$. F1 P2Y₄$^{+/-}$ female mice were then crossed with wild-type CD1 male mice. The frequency of the F2 newborn mice in terms of sex and genotype were as expected (Fig. 2B). After a series of crossings between F2 P2Y₄$^{0/-}$ male mice and F1 P2Y₄$^{+/-}$ female mice, the offspring comprised 120 male mice and 79 female mice. Among the male mice, 45.8% had a $P2Y_4^{0/+}$ genotype and 54.2% had a $P2Y_4^{0/-}$ genotype. This frequency was not statistically different from the theoretical 50%/50% repartition. On the other hand, among the female mice, we observed 62% P2Y₄$^{+/-}$ female mice and 38% P2Y₄$^{-/-}$ mice, which is statistically significantly different (p = 0.0003) from the expected repartition.

P2Y₄-deficient mice were not distinguishable from wild-type mice. Their behavior in the cage seemed normal. They were fertile and their growth was indistinguishable from that of wild-type mice (data not shown). Classic histological examination did not reveal differences in any of tested organs (i.e., brain, heart, lung, intestine, liver, salivary gland, pancreas, stomach, spleen, thymus, thyroid, adrenal gland, kidney, bladder, testis, prostate gland, seminal gland, ovary, uterus, or vagina) (not shown).

To confirm the targeting of the $P2Y_4$ gene, we assayed the expression of the corresponding mRNA by RT-PCR in liver, stomach, and intestine, the organs in which the highest expression was found previously (Suarez-Huerta et al., 2001). The expected 336-bp signal was detected in tissues of wild-type mice but not when RNA were extracted from P2Y$^{0/-}$

animals, whereas HPRT expression was detected in all tissues independent of mouse genotype (Fig. 3).

Study of Cl⁻ Secretory Response to Nucleotides in Isolated Jejunum. Extracellular nucleotides regulate chloride secretion in many epithelia, including the jejunum. In particular, UTP and ATP increase chloride secretion in the mouse jejunum; this response is maintained in P2Y₂$^{-/-}$ mice (Cressman et al., 1999). We therefore investigated whether the P2Y₄ receptor could be involved in such control. The addition of UTP (100 μM) to the apical solution induced an increase in SCC in 5 of 8 jejuna from P2Y₄$^{0/+}$ mice. But no increase was ever observed in jejuna from P2Y₄$^{0/-}$ mice that were still responsive to forskolin (Fig. 4). This difference between the two groups was statistically significant even though the control group included "nonresponder" tissues (Table 1). In similar experiments, we tested the effect of ATP (100 μM), the other agonist of the P2Y₄ receptor. An increase in SCC was similarly observed in jejuna from P2Y₄$^{0/+}$ mice (five of eight responses) but not in jejuna from P2Y₄$^{0/-}$ mice
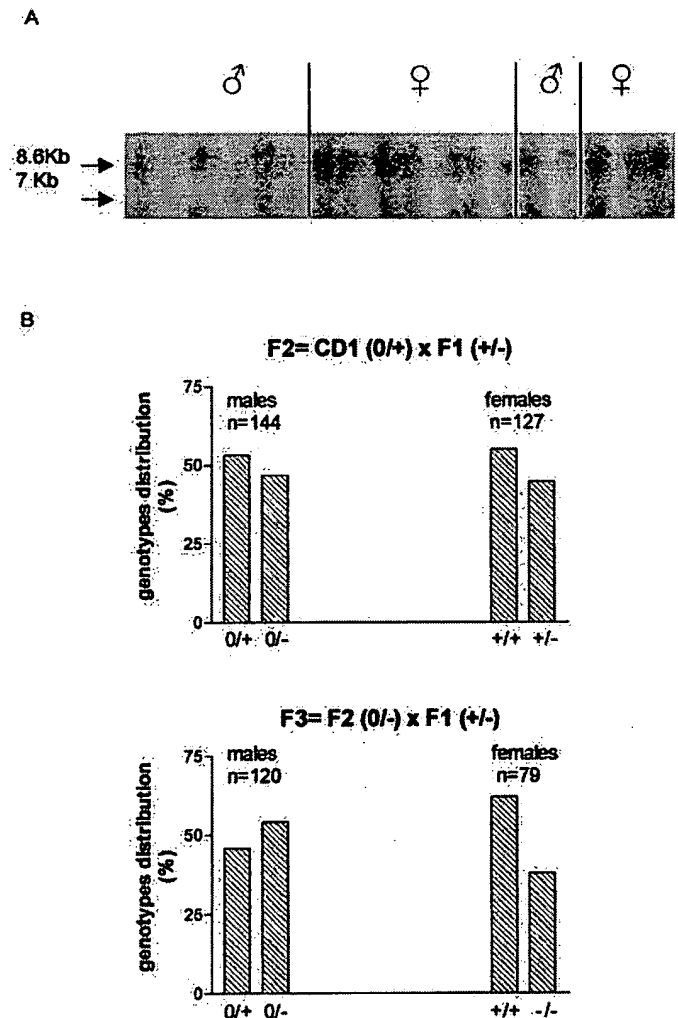
Fig. 2. Repartition of newborn mice in terms of sex and genotype. A, Southern blot analysis of genomic DNA extracted from F1 newborn mice. Chimeric mice were crossed with CD1 wild-type female mice. Genomic DNA of offspring was analyzed as described under *Materials and Methods*. B, histogram of the repartition of sex and genotype of mice as function of crossing. After crossing, the genotype of offspring was determined by analysis of genomic DNA as described under *Materials and Methods*.
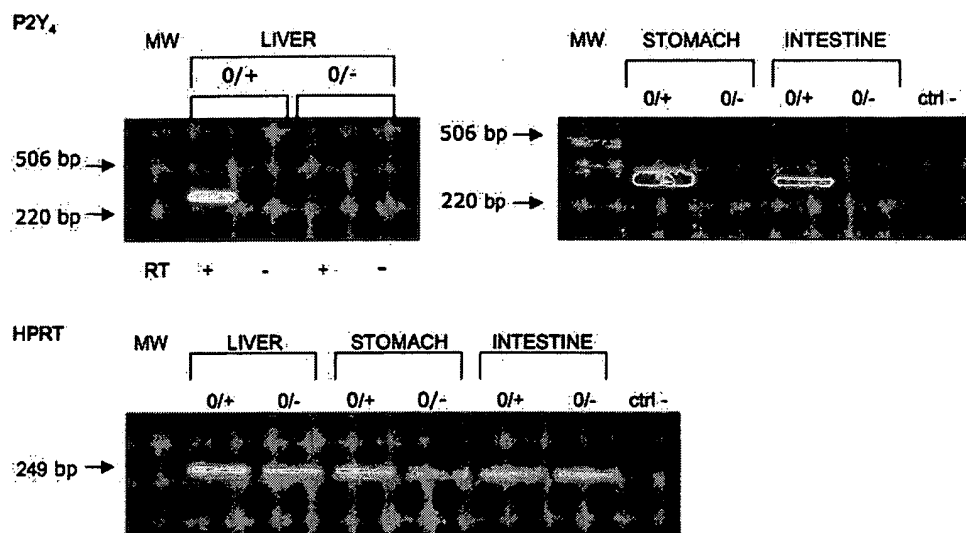
P2Y₄

HPRT

Fig. 3. Detection of P2Y₄ mRNA in P2Y₄^{O/+} or P2Y₄^{O/−} mouse tissues by RT-PCR. The extraction of RNA and the OneStep RT-PCR were performed as described under *Materials and Methods*. PCR products of 336 bp for P2Y₄ and 249 bp for HPRT are visualized under UV after electrophoresis on a 1.5% agarose gel and ethidium bromide coloration. RT −, controls for genomic DNA contamination (i.e., PCR without reverse transcription).

(Fig. 5 and Table 1). To exclude the possibility that the latter effect could be linked to extracellular degradation of ATP into adenosine by apical 5′-nucleotidase, these experiments were repeated in the presence of 8-*p*-SPT, a nonspecific antagonist of adenosine receptors. In these conditions, apical ATP still induced an increase in SCC in seven of eight jejuna from P2Y₄^{O/+} mice but not in jejuna from null mice (Table 1).

On the other hand, when both groups of jejuna from control and knockout mice (n = 24 in each group) were compared, no

statistically significant difference was observed in mean baseline SCC or in mean SCC after addition of the different drugs (phlorizin, indomethacin, and forskolin) except the purinergic agonists (Table 2). This indicates that P2Y₄ deletion failed to influence the different components of the SCC other than the chloride secretion response to apical purinergic agonists (i.e., in particular, the Na-glucose component and the various parts of the Cl⁻ component of SCC) (Table 2).
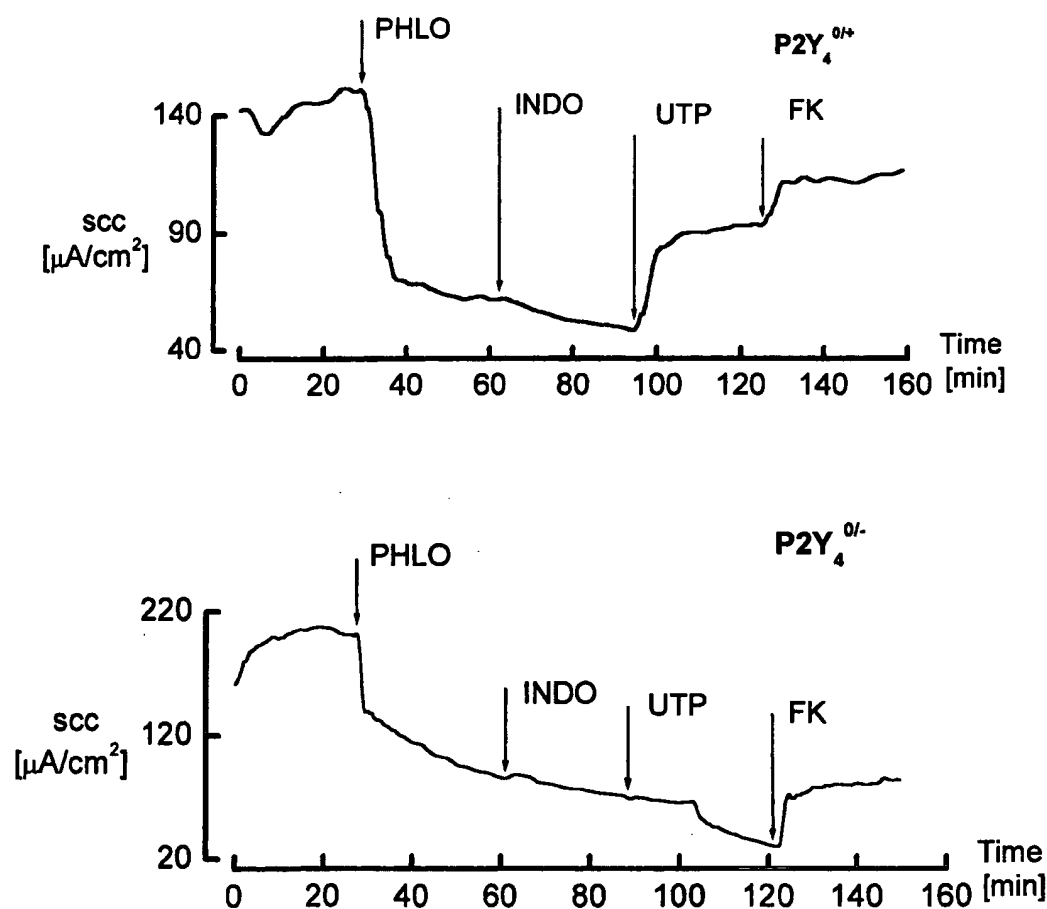
In control mice, the mean increases in SCC after addition

Fig. 4. Stimulation of Cl⁻ secretion by UTP in the jejunum of control and P2Y₄ knockout mice. Sodium reabsorption was inhibited by adding 1 mM phlorizin (PHLO) to the apical solution. Indomethacin (INDO; 100 μM) was added to both sides. Top, time course of SSC in an experiment with a control mouse; bottom, an experiment with a knockout mouse. At the end of the experiment, the stimulation of Cl⁻ secretion by forskolin (10 μM in the basolateral bath) was tested.

TABLE 1

Increase in SCC in mice jejuna induced by purinergic agonists

The mean increase (± S.E.M.) in SCC (μA/cm^2) was calculated from recordings illustrated in Figs. 4 and 5 as the difference in SCC immediately before and 20 min after stimulation with the different purinergic agonists The mean response computed for control tissues includes also the "non-responders"; n = 8 in each group.

Stimulus	n	P2Y₄$^{0/+}$	P2Y₄$^{0/-}$	Significance
UTP	8	17 ± 5	2 ± 1	$P<0.01$
ATP	8	28 ± 8*	3 ± 2	$P<0.01$
ATP + 8-p-SPT	8	27 ± 6*	2 ± 2	$P<0.001$

* $P > 0.2$ compared with the increase in current induced by UTP in P2Y₄$^{0/+}$ mice.

of UTP, ATP, or ATP in the presence of 8-p-SPT were not statistically significantly different from each other, in agreement with the known equipotency of these agonists for the murine P2Y₄ receptor.

Discussion

The actions of extracellular nucleotides are mediated by two classes of receptors: P2X receptors, which have an intrinsic activity of ion channels, and P2Y receptors coupled to G proteins. Pharmacologically, P2Y receptors can be subdivided into adenine nucleotide-preferring receptors responding mainly to ADP (P2Y$_1$, P2Y$_{12}$, P2Y$_{13}$) and ATP (P2Y$_{11}$), uracil nucleotide-preferring receptors (human P2Y$_4$, P2Y$_6$) responding to UTP and UDP, respectively, and receptors of mixed selectivity activated by both ATP and UTP (P2Y$_2$, rodent P2Y$_4$). One important action of extracellular ATP and UTP is to stimulate the transepithelial secretion of chloride as a result of increased apical permeability (Knowles et al.,

1991). This process is mediated by an inositol triphosphate-mediated increase in cytosolic Ca^{2+} that induces the opening of outwardly rectifying chloride channels (Clarke et al., 1994). In some instances, however, it is caused by the activation of the cystic fibrosis transmembrane conductance regulator (CFTR). Calcium-induced activation of CFTR could result from increased insertion into the apical membrane (Cantiello et al., 1994; Atia et al., 1999; Cuffe et al., 2000). Thus, apical chloride permeability is caused by multiple chloride channels and seems controlled by multiple P2Y receptors. For instance, whereas the chloride secretory response to UTP was completely abolished in the trachea of P2Y$_2$-null mice, the response to UTP was maintained partially in the gallbladder and completely in the jejunum (Cressman et al., 1999). Cressman et al. (1999) suggested that the jejunal response to UTP could be transduced by the P2Y$_4$ receptor, the mRNA of which is indeed expressed in small intestine (Lazarowski et al., 2001; Suarez-Huerta et al., 2001). However, an alternative explanation is that the P2Y$_2$ receptor is physiologically involved in the jejunal response but that compensation by P2Y$_4$ occurs after its inactivation. For instance, it has been recently shown that mice lacking all three β-adrenergic receptors (β_1, β_2, β_3) cannot increase thermogenesis and become severely obese during overfeeding, whereas gene inactivation of individual β-adrenergic receptors had no significant effect (Bachman et al., 2002). We have now generated P2Y$_4$-null mice. The invalidation was confirmed by loss of expression of the mRNA in intestine, stomach, and liver. These mutant mice are grossly normal, and the only pheno-
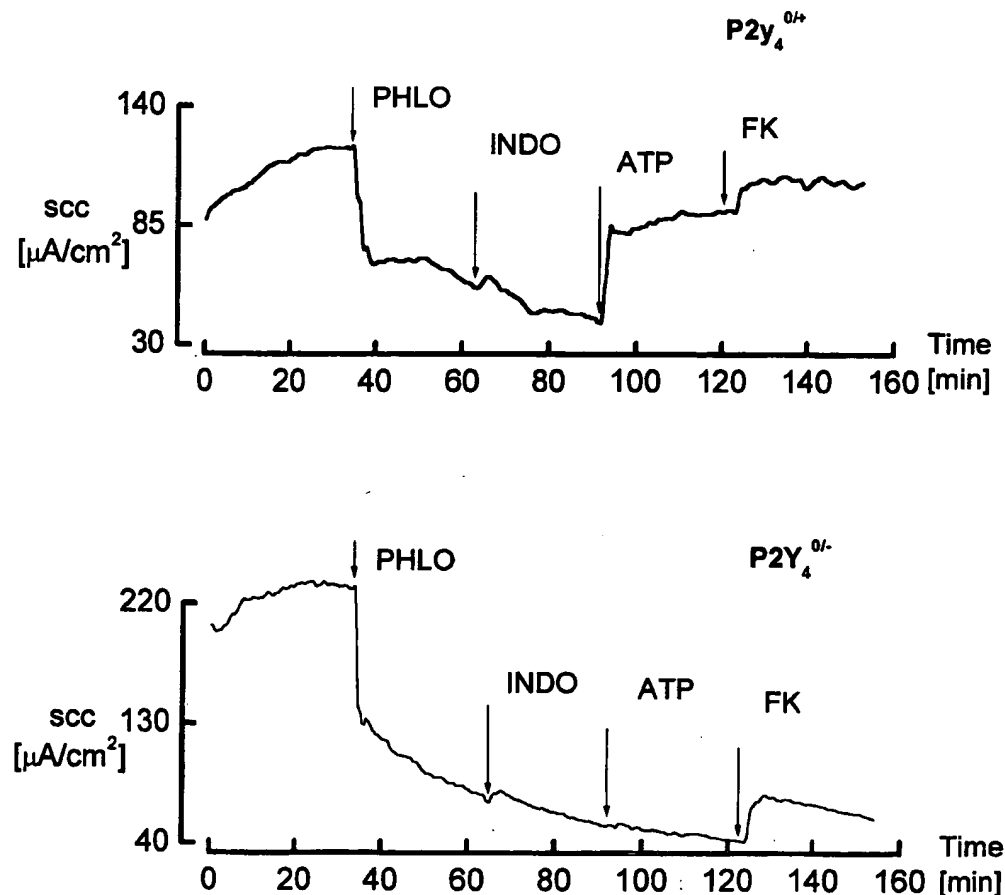
P2y₄$^{0/+}$

P2Y₄$^{0/-}$

Fig. 5. Stimulation of Cl$^-$ secretion by ATP in the jejunum of control and P2Y₄ knockout mice. The protocol was similar to that of Fig. 4, except that ATP (100 μM) instead of UTP was used as stimulatory agent.

TABLE 2
SCC of mice jejuna after addition of the various drugs

The mean SCC ($\mu A/cm^2$) ± S.E.M. was calculated from recordings illustrated in Fig. 4 and 5 as the baseline SCC 20 min after the beginning of the experiment and 20 min after the various drugs added. Because in the case of forskolin, the tissue has been previously exposed to a purinergic agonist with or without an increase in SCC, we provide rather the mean increase in SCC calculated as the difference in SCC immediately before and 20 min after forskolin addition. $n = 24$ for each group.

Drug	$P2Y_4^{0/+}$	$P2Y_4^{0/-}$	Significance
None	181 ± 16	168 ± 13	$P > 0.5$
Phlorizin	76 ± 8	63 ± 5	$P > 0.1$
Indomethacin	64 ± 6	52 ± 5	$P > 0.1$
Forskolin	31 ± 3	40 ± 5	$P > 0.1$

typic abnormality that we have detected so far is the disappearance of the jejunal chloride secretory response to UTP and ATP, thus confirming the hypothesis of Cressman et al. (1999). According to these authors, the increased chloride secretion induced by UTP is observed in only about two thirds of the normal mice, perhaps as a result of ATP release during mounting of the tissue in the chamber and receptor occupancy or desensitization. The mean increase in SSC observed here in control mice was similar with UTP and ATP or ATP in the presence of 8-p-SPT, although smaller but more sustained than that reported by Cressman et al. (1999). This difference could be related to the different strains of mice used. We have tested the effect of 8-p-SPT because ATP has been reported to stimulate CFTR via its local degradation into adenosine with subsequent activation of the A_{2B} receptor (Huang et al., 2001; Matsuoka et al., 2002): the complete disappearance of the ATP response in $P2Y_4^{0/-}$ mice indicates that this mechanism does not operate in mouse jejunum, despite the fact that we observed a response to exogenous adenosine (E. Ghanem and R. Beauwens, unpublished observations) at variance with Cressman et al. (1999).

Provided they can be extrapolated to humans, our results suggest that the $P2Y_4$ receptor should be considered a potential pharmacotherapeutic target for treatment of cystic fibrosis and diarrhea. Indeed cystic fibrosis is associated with gastrointestinal abnormalities. Ten to 15% of CFTR-newborns have meconium ileus. A major problem is long-term malabsorption resulting from both pancreatic insufficiency and the thick layer of highly viscous mucus that coats the absorptive surface and constitutes a diffusion barrier for nutrients. Finally, a significant fraction of adult patients suffer from distal intestinal obstruction. By analogy with the use of aerosolized $P2Y_2$ agonists to hydrate the airway mucus (Kellerman, 2002), oral $P2Y_4$ receptor agonists might be used to stimulate the jejunal secretion of electrolytes and water. The UTP effect on the murine jejunum is abolished in CFTR-null mice, which are completely deficient in that protein (Lazarowski et al., 2001). However the major genetic defect responsible for human cystic fibrosis is the ΔF508 mutation, which is associated with inefficient trafficking to the membrane and decreased activity. It is known, however, that ΔF508 CFTR is significantly expressed in human jejunum (Kalin et al., 1999). The activity of the CFTR can be increased not only by cAMP but also by G proteins and some calcium-dependent protein kinases. In particular, pharmacological activation of the ΔF508 CFTR has been demonstrated in the jejunum of transgenic mice expressing the mutated protein (Steagall and Drumm, 1999). Moreover, thapsigargin, an inhibitor of the endoplasmic reticulum calcium pump, has re-

cently been shown to increase plasma membrane ΔF508 CFTR (Egan et al., 2002). It will therefore be of interest to establish whether the trafficking and/or the activity of the ΔF508 CFTR can be modulated by exposure to UTP. Alternatively, a $P2Y_4$ antagonist might be of value in the treatment of the secretory diarrhea induced by some enteropathogenic bacteria, which have indeed been shown to induce nucleotide release (McNamara et al., 2001; Crane et al., 2002). Additional phenotypic studies of $P2Y_4$-null mice, focused on inner-ear function and angiogenesis, are currently underway.

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